Regulation of dauer larva development in *Caenorhabditis* elegans by daf-18, a homologue of the tumour suppressor PTEN

Jean-Pierre Rouault*†, Patricia E. Kuwabara‡†, Olga M. Sinilnikova§, Laurent Duret[¶], Danielle Thierry-Mieg[¥] and Marc Billaud[§]

The tumour suppressor gene PTEN (also called MMAC1 or TEP1) is somatically mutated in a variety of cancer types [1-4]. In addition, germline mutation of PTEN is responsible for two dominantly inherited, related cancer syndromes called Cowden disease and Bannayan-Ruvalcaba-Riley syndrome [4]. PTEN encodes a dual-specificity phosphatase that inhibits cell spreading and migration partly by inhibiting integrin-mediated signalling [5-7]. Furthermore, PTEN regulates the levels of phosphatidylinositol 3,4,5trisphosphate (PIP₂) by specifically dephosphorylating position 3 on the inositol ring [8]. We report here that the dauer formation gene daf-18 is the Caenorhabditis elegans homologue of PTEN. DAF-18 is a component of the insulin-like signalling pathway controlling entry into diapause and adult longevity that is regulated by the DAF-2 receptor tyrosine kinase and the AGE-1 PI 3-kinase [9]. Others have shown that mutation of daf-18 suppresses the life extension and constitutive dauer formation associated with daf-2 or age-1 mutants. Similarly, we show that inactivation of daf-18 by RNA-mediated interference mimics this suppression, and that a wild-type daf-18 transgene rescues the dauer defect. These results indicate that PTEN/DAF-18 antagonizes the DAF-2-AGE-1 pathway, perhaps by catalyzing dephosphorylation of the PIP₃ generated by AGE-1. These data further support the notion that mutations of PTEN contribute to the development of human neoplasia through an aberrant activation of the PI 3-kinase signalling cascade.

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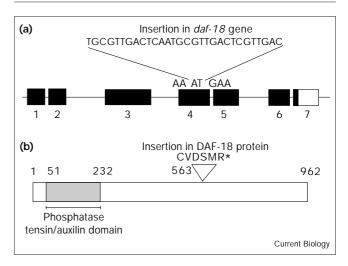
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Results and discussion

We identified a cosmid (T07A9) and an expressed sequence tag (EST; yk400b8) that encode a putative C. elegans homologue of the mammalian PTEN gene (Figure 1a). The C. elegans gene is located on the left arm of chromosome IV, between daf-1 and opu-18. Complete sequence analysis of the yk400b8 cDNA (the cDNA was kindly provided by Yuji Kohara) revealed a 962 amino-acid open-reading frame (see Supplementary material published with this paper on the internet); a 180 amino-acid region of this predicted protein has significant sequence similarity (46% identity) with the tensin/phosphatase domain of PTEN (Figure 1b). A multiple sequence alignment of PTEN homologues shows that the phosphatase signature motifs known to be essential for catalysis are conserved in the C. elegans PTEN homologue (see Supplementary material); for example, one conserved

Figure 1



(a) Physical map of the C. elegans PTEN gene (Genbank accession number AF036706). The exon-intron structure was determined by comparing genomic DNA and cDNA sequences; the seven exons are depicted as boxes with shading indicating the coding sequence. The sequence of the cDNA (EMBL accession number AJ131181) showed that the 3' end of exon 5 is 9 bp upstream of the position predicted by Genefinder (T07A9.6, accession number 044405). The *daf-18*(*e1375*) mutation was identified by sequencing polymerase chain reaction products amplified from genomic DNA isolated from daf-18(e1375) mutant animals. This mutation was found to consist of an insertion of 30 nucleotides combined with the deletion of 2 nucleotides within exon 4. The insertion is composed of two repeats of 11 nucleotides (TGCGTTGACTC) and of an incomplete repeat GTTGAC (b) Schematic representation of the C. elegans PTEN protein. The daf-18 mutation results in the addition of 6 amino acids and the formation of a stop codon (asterisk) after amino acid 563.

Table 1 Effects of PTEN/daf-18 dsRNA injection on daf-2 dauer formation.

RNAi	Phenotype of progeny at 25.5°C (%)				
	N	Adult	Dauer	Dead	
Uninjected	1340	0	99	1	
PTEN/daf-18	841	68	29	3	
tra-2	685	0	98	2	

PTEN/daf-18 dsRNA was synthesized using a subclone of the yk400b8 cDNA as a template, and 2 mg/ml of this dsRNA was injected into six adult daf-2(e1368) hermaphrodites. Control daf-2(e1368) animals were left uninjected or injected with the tra-2 dsRNA. The animals were grown at the permissive temperature (15°C) then shifted to 25.5°C after injection. They were transferred to a fresh plate 12-18 h after injection and then subsequently transferred at 24 h intervals. All the F1 progeny born from injected animals were scored for phenotype, therefore the effect of RNAi was underestimated as animals produced by eggs not affected by RNAi at the time of injection were also scored for phenotype. N represents the total number of F1 animals scored after injection.

sequence is HCXAGXXR (in single-letter amino-acid code, where X represents any amino acid), which contains the cysteine involved in forming the thiol-phosphate bond with the substrate [10]. Constructing a phylogenetic tree of the tensin family shows that the C. elegans PTEN homologue is more closely related to mammalian PTEN than to other members of this family (see Supplementary material). Therefore, the high conservation of the catalytic core sequence across evolution suggests that C. elegans PTEN has an enzymatic activity similar to its mammalian counterparts.

Given the capacity of PTEN to dephosphorylate PIP₃ at position 3 on the inositol ring [8], we reasoned that the C. elegans PTEN homologue might antagonize the function of the PI 3-kinase AGE-1 [11]. The age-1 gene is involved in the genetic pathway controlling the formation of dauer larvae, the rate of ageing and the resistance to oxidative and UV stress and to thermotolerance [11-16]; mutations in age-1 lead to constitutive dauer formation [13–15]. Therefore, a simple model would predict that a mutation in the C. elegans PTEN homologue should lead to a dauer-defective phenotype — the opposite effect of age-1 mutants. Previous studies have shown that a daf-18 mutant is defective in dauer formation, and epistatic analyses have demonstrated that mutation of daf-18 inhibits dauer formation caused by the age-1(m333) mutant and suppresses life-span extension caused by age-1(hx546) [12–15]. Furthermore, the daf-18 gene is located on chromosome IV and maps to a region compatible with the position of the PTEN homologue. On the basis of these arguments, daf-18 was considered to be an excellent candidate for the C. elegans PTEN. We therefore sequenced the PTEN gene in the daf-18

Table 2 Effects of PTEN/daf-18 dsRNA injection on age-1 dauer formation

		Phenotype of progeny at 20°C (%)				
RNAi	N	Adult	Dauer	Dead		
Uninjected	361	0	100	0		
PTEN/daf-18	746	92	7	1		

In this series of experiments, 18 non-dauer animals from an age-1(mg44)/mnC1 strain were injected with the PTEN/daf-18 dsRNA described in Table 1. The animals were either age-1(mg44)/mnC1 or age-1/age-1 (m+z-, indicating the presence of the maternal and absence of the zygotic wild-type alleles) in genotype, because the age-1 mutation is maternally rescued. To distinguish between these alternative genotypes, the F1 progeny were analyzed: age-1(mg44)/mnC1 animals produce a fraction of homozygous uncoordinated mnC1 progeny, whereas age-1/age-1 (m+z-) parents do not produce mnC1 progeny. In this study, 8 out of 18 of the injected animals were age-1/age-1 (m^+z^-). N represents the total number of progeny scored from the 8 injected age-1/age-1 (m+z-) parents.

mutant strain CB1375 and found an insertion of 30 base pairs in exon 4 (Figure 1a) that results in the addition of six amino acids followed by a premature stop codon immediately after amino acid 563 (Figure 1b). This mutation falls outside the phosphatase domain and is predicted either to truncate the PTEN/DAF-18 protein or to lead to destabilization of the transcript or protein.

The daf-2 gene encodes a member of the insulin receptor family that is implicated in the genetic pathway controlling dauer formation and the rate of ageing [13-20]. Genetic epistasis interactions place the daf-2(e1370, m65) mutant upstream of daf-18 for life-span determination [14,15]; the order of these two genes in the pathway regulating dauer formation is somewhat ambiguous, however, because daf-2(e1370) daf-18 double mutants can enter, and rapidly exit, the dauer state [14,21]. To explore the role of PTEN/daf-18 in the daf-2-mediated control of diapause, we used RNA interference (RNAi) [22] to inactivate PTEN/daf-18 expression in daf-2(e1368) and age-1(mg44) mutants. As shown in Table 1, daf-2 mutants from parents injected with the unrelated tra-2 doublestranded interfering RNA (dsRNA) and grown at the restrictive temperature arrested as dauers, whereas 68% of those from daf-2 parents injected with PTEN/daf-18 dsRNA gave rise to adults. Similarly, RNAi inhibition of PTEN/daf-18 in age-1(mg44) mutants resulted in an almost complete rescue of the age-1 phenotype: 92% of the progeny of injected worms proceeded to the adult stage (Table 2). Finally, we showed that a wild-type daf-18 transgene is capable of rescuing daf-18 in a daf-2(e1368) daf-18 double mutant, which is dauer-defective. In this mutant background, the ability of a transgene to restore dauer formation at the restrictive temperature is an indicator of daf-18 rescue. As shown in Table 3, 30% of

Table 3 Expression of PTEN/daf-18 rescues the dauer-defective phenotype in the daf-2(e1368) daf-18(e1375) double mutant.

	Phenotype of progeny at 25.5°C (%)			
Transgenes	N	Roller adult	Roller dauer	
rol6	1,166	11	1 <	
PTEN/daf18 + rol6	731	13	30	

The double mutant strain daf-2(e1368) daf-18(e1375) was constructed using standard genetic methods. These double-mutant animals developed as viable adults at the restrictive temperature (25.5°C). The dominant transformation marker rol-6 (su1006) was singly injected in the syncytial gonad or co-injected with 7.6 kb of genomic DNA containing the PTEN/daf-18 gene. Transgenic roller animals were picked, and the phenotype was scored (roller adult versus roller dauer) after shifting the temperature from 15°C to 25.5°C. The transgene is an extrachromosomal array that imperfectly segregates in mitosis and meiosis, thus explaining the variation of the transmission rate.

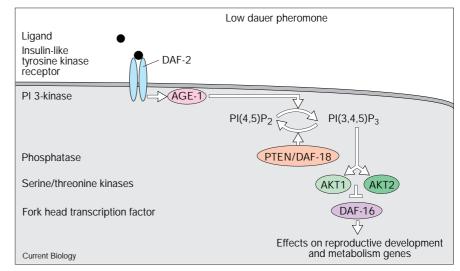
daf-2(e1368) daf-18 double mutants carrying a transgene consisting of the wild-type daf-18 gene and the visible marker rol-6 developed into dauers at the restrictive temperature (25.5°C), whereas control animals carrying only a rol-6 transgene were unable to form dauers. Overall, these results demonstrate that PTEN/daf-18 is epistatic to daf-2 and age-1 for the control of dauer formation and sustain the notion that PTEN/DAF-18 has a critical function downstream of the AGE-1 PI 3-kinase.

On the basis of these results, we propose that PTEN/DAF-18 negatively controls the DAF-2-AGE-1-AKT pathway and ensures a transient activation of the kinase AKT upon stimulation of DAF-2 by its cognate ligand (Figure 2). The high conservation of the core enzymatic domain between PTEN/DAF-18 and mammalian PTEN supports the

notion that these enzymes recognize similar substrates and thereby favours the idea that PTEN/DAF-18 antagonizes AGE-1 PI 3-kinase via its PIP₃ 3-phosphatase activity (Figure 2). If so, then PTEN/daf-18 lies downstream of age-1 and upstream of akt and the fork head transcription factor daf-16 in the daf-2-mediated decision to enter diapause. The epistatic ordering of genes involved in the daf-2 pathway that controls dauer formation can be interpreted as a linear signalling cascade whereby daf-2, age-1, daf-18, akt and daf-16 act within the same cell. Genetic mosaic analyses have recently challenged this straightforward conception, however, by revealing that daf-2 function in the control of dauer development and longevity is not cellautonomous [23]. Nevertheless, it remains very likely that age-1, PTEN/daf-18 and akt operate within the same cells. The observation that PTEN/daf-18 suppresses dauer formation of null alleles of age-1, such as the mg44 mutation (which is predicted to truncate the PI 3-kinase lipid kinase domain [11]), suggests that PIP3 might also be produced in an AGE-1-independent manner and that disruption of DAF-18 would raise the level of PIP₃ above the threshold required for the activation of AKT. In addition, the F1 progeny of animals injected with PTEN/daf-18 RNAi displayed defects in vulval development, thus indicating that PTEN/daf-18 is likely to have a role during vulval morphogenesis. The nature of the *daf-18(e1375)* mutation suggests that it might be hypomorphic and so the absence of other PTEN/daf-18 alleles raises the possibility that a null mutation in PTEN/daf-18 might be lethal. Although experiments with PTEN/daf-18 RNAi have so far failed to uncover any significantly elevated lethality, it is possible that RNAi is unable to block PTEN/daf-18 expression completely. Therefore, it remains important to obtain additional PTEN/daf-18 alleles in order to determine whether this gene plays an essential role in *C. elegans* development.

Figure 2

Model for the function of PTEN/DAF-18 during C. elegans dauer development. The normal life cycle of C. elegans occurs when the dauer pheromone is present at low concentration. Reduced levels of the pheromone result in the activation of the DAF-2 receptor tyrosine kinase via the binding of a putative insulin-like ligand. Activated DAF-2 promotes the stimulation of AGE-1 and the ensuing production of PIP3 which allows the recruitment of AKT (AKT1 and AKT2) to the plasma membrane and their subsequent activation. AKT might directly phosphorylate the DAF-16 transcription factor and thereby relieve its repressor function. In this model, PTEN/DAF-18 would antagonize AGE-1 function by catalyzing the dephosphorylation of PIP3, thereby ensuring that activation of the DAF-2-AGE-1 pathway is transient.



Dysregulation of the PI 3-kinase pathway has clearly been involved in the transformation process mediated by various oncogenes [24]. Also, amplification of PIK3CA, which encodes the catalytic subunit of PI 3-kinase, has been characterized in a frequent proportion of ovarian cancers, and amplification of Akt2 has been characterized in a fraction of ovarian, pancreatic and breast cancers [25-27]. Our data underscore the key role that PTEN/DAF-18 might have in the regulation of the PI 3-kinase-AKT pathway and strongly support the notion that inactivating mutations of PTEN in human tumours are likely to result in an aberrant stimulation of Akt and downstream effectors — a model substantiated by several recent studies [28–30]. While this work was being submitted for publication, Ogg and Ruvkun [31] also reported similar findings, namely that DAF-18 is a C. elegans PTEN homologue that acts in the insulin-like receptor signalling pathway.

Supplementary material

The sequence of the predicted PTEN homologue protein deduced from the sequence of the full-length cDNA yk400b8, an alignment of the tensin/phosphatase domain with members of the PTEN family, a phylogenetic tree of the tensin family, and additional methodological details are published with this paper on the internet.

Acknowledgements

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Supplementary material

Regulation of dauer larva development in *Caenorhabditis elegans* by *daf-18*, a homologue of the tumour suppressor *PTEN*

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Figure S1

(a) 0.05 MVTPPPDVPSTSTRSMARDLOENPNROPGEPRVSEPYHNSIVERIRHIFR TAVSSNRCRTEYQNIDLDCAYITDRIIAIGYPATGIEANFRNSKVQTQQF PTEN C. elegans LTRRHGKGNVKVFNLRGGYYYDADNFDGNVICFDMTDHHPPSLELMAPFC REAKEWLEADDKHVIAVHCKAGKGRTGVMICALLIYINFYPSPRQILDYY 100 SIIRTKNNKGVTIPSQRRYIYYYHKLRERELNYLPLRMQLIGVYVERPPK PTEN2 human TWGGGSKIKVEVGNGSTILFKPDPLIISKSNHORERATWLNNCDTPNEFD TGEQKYHGFVSKRAYCFMVPEDAPVFVEGDVRIDIREIGFLKKFSDGKIG ${\tt HVWFNTMFACDGGLNGGHFEYVDKTQPYIGDDTSIGRKNGMRRNETPMRK}$ 100 PTEN rat IDPETGNEFESPWOIVNPPGLEKHITEEOAMENYTNYGMIPPRYTISKIL HEKHEKGIVKDDYNDRKLPMGDKSYTESGKSGDIRGVGGPFEIPYKAEEH VLTFPVYEMDRALKSKDLNNGMKLHVVLRCVDTRDSKMMEKSEVFGNLAF 100 HNESTRRLQALTQMNPKWRPEPCAFGSKGAEMHYPPSVRYSSNDGKYNGA PTEN mouse CSENLVSDFFEHRNIAVLNRYCRYFYKQRSTSRSRYPRKFRYCPLIKKHF 95 YIPADTDDVDENGQPFFHSPEHYIKEQEKIDAEKAAKGIENTGPSTSGSS APGTIKKTEASQSDKVKPATEDELPPARLPDNVRRFPVVGVDFENPEEES ^IPTFN human CEHKTVESIAGFEPLEHLFHESYHPNTAGNMLRQDYHTDSEVKIAEQEAK ${\tt AFVDQLLNGQGVLQEFMKQFKVPSDNSFADYVTGQAEVFKAQIALLEQSE}$ DFQRVQANAEEVDLEHTLGEAFERFGHVVEESNGSSKNPKALKTREQMVK GAK human ETGKDTQKTRNHVLLHLEANHRVQIERRETCPELHPEDKIPRIAHFSENS 100 FSDSNFDQAIYL (b) GAK rat PTEN rat 100 PTEN human PTEN mouse VSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLDSKH-KNHYKI VSRNKRRYQEDGFDLDLTYIYPNIIAMGFPAERLEGVYRNNIDDVVRFLDSKH-KNHYKI PTFN2 human VSBNKBBAUGEDTUT.AAATTAWGEDAESTEGAAANTUDAAAETUSKH-KNHAKT PTEN C. elegans **AUXILIN** bovine PTEN rat YNLCAERHYDTAKFNCRVAOYPFEDHNPPOLELIKPFCEDLDOWLSEDDNHVAAIHCKAG INLCABRHYDTAKYNCKYAQYPFEDHNPPQLELIKPFCEDLDQWLSEDDNHVAAIHCKAG
YNLCABRHYDTAKYNCKVAQYPFEDHNPPQLELIKPFCEDLDQWLSEDDNHVAAIHCKAG
YNLCABRHYDTAKFNCRVAQYPFEDHNPPQLELIKPFCEDLDQWLSEDDNHVAAIHCKAG
HNLCABRHYDTAKSNYRVAQYPFEDHNPPQLELIKPFCEDLDQWLSEDDNHVAAIHCKAG PTEN human F46F11.3 C. elegans PTEN C. elegans PTEN rat PTEN human PTEN mouse KGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPSQRRYVYYYSYLLKNHLDYKGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPSQRRYVYYYSYLLKNHLDY TENSIN chicken KGRTGVMICAYLLHRGKFLKAQEALDFYGEVRTRDKKGVTIPSQRRYVYYYSYLLKNHLDY PTFN2 human KGRTGIMIYAYI.I.HRGKFI.KAOEAI.DFYGEVRTRDKKGVTIPSORRYVYYYSYI.VKNHI.DY PTEN C. elegans

(a) Sequence of the predicted PTEN homologue protein in *C. elegans* deduced from the sequence of the full-length cDNA yk400b8.
(b) Alignment of the tensin/phosphatase domain from members of the PTEN family. The alignment was computed with CLUSTALW. Identities are symbolised by a star and similarities by a dot. Dashes indicate skipped residues. (c) Phylogenetic tree of the tensin family. This tree was constructed using the neighbor joining method [S1], based on the alignment of the amino-terminal part of the tensin domain

(175 sites), which is conserved in all members of the tensin family. Bootstrap values (500 replicates) are indicated. SWISSPROT accession numbers are as follows: for PTEN: human (000633), mouse (008586), rat (054857), *C. elegans* (T07A9.6, O44405); PTEN2: human (043460, 014781); GAK (cyclin G-associated kinase): human (014976), rat (P97874); TENSIN: chicken (Q04205); F46F11.3: *C. elegans* hypothetical 30.3 kDa protein F46F11.3 (P91301); AUXILIN: bovine (Q27974).

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Supplementary materials and methods

DNA sequencing of the PTEN/daf-18 gene Worms (N2 and CB1375 daf-18(e1375) strains) were lysed in 20 μ l of lysis buffer (Tris-HCl pH 8.3, KCl 50 mM, MgCl $_2$ 2.5 mM, NP40 0.45%, Tween 20 0.01%, gelatin 0.01% and 1 ml proteinase K at 2 mg/ml) for 1 h at 60°C. The reaction was stopped by incubation at 95°C for

15 min. The *PTEN/daf-18* gene was PCR amplified with the Expand Hi-Fidelity polymerase (Boehringer Mannheim) using five different primer pairs (P6/P19; P5/P7; P1/P13; P10/P16; P15/P8) and 2 μl of worm lysates. P1: 5'-AAATAGTGAATCCTCCTG-3'; P5: 5'-ACTCAA-CAATTCTGACC-3'; P6: 5'-ATTCACACTTCACC-3'; P7: 5'-ATTTAGCAGCCTTATCTC-3'; P8: 5'-ATTTTCAGATTCGTCGG-3';

P10: 5'-CATAAACCTACTTTTTCAAC-3'; P13: 5'-CTCCATTAT-ACTTTCCATC-3'; P15: 5'-CTTTTTATATTATATGCGGG-3' P16: 5'-GATACAAGAAAATAGTCACC-3'; P19: 5'-GATTTGGAGATTATGAGAG-3'. Sequence data collection and analysis were performed on a DNA sequencer (Model 373; Applied Biosystems) by Genome Express (Grenoble, France).

RNAi experiments

DNA templates for in vitro transcription were linearized by digesting a subclone of the yk400b8 cDNA clone with KpnI or Spel. 2 µg of linearized DNA template were included in an RNA synthesis reaction using either T3 or T7 RNA polymerase, as directed by the manufacturer (Boehringer-Mannheim), to generate sense or antisense RNA, respectively. At the end of the reaction, the DNA template was digested with RNase-free DNase, extracted with phenol: chloroform (1:1), and precipitated with isopropanol. The RNAs were resuspended in TE (10 mM Tris-Cl pH 7.5; 1 mM EDTA) and mixed in equal molar ratios to generate double-stranded RNA (dsRNA). The dsRNA was injected into the germline syncytium of adult hermaphrodites using the methods previously described (see references in [S1]). Injected daf-2(e1368) mutants were raised at the restrictive temperature of 25.5°C, whereas injected age-1(mg44) mutants were raised at 20°C. Injected animals were transferred to fresh plates daily and the phenotypes of their F1 progeny were scored by dissecting microscopy. Control injections were performed using dsRNA corresponding to the sex-determining gene tra-2.

Transgenic strains

The 7.6 kb of genomic DNA containing the PTEN/daf-18 gene, including 1.3 kb of upstream regulatory sequences and 1.6 kb of 3' untranslated region, was PCR amplified with the Expand Long polymerase, as directed by the manufacturer (Boerhinger Mannheim) and using one primer pair: ePTEN-F: 5'-GATCGCGGCCGCGATCCAA-AAATCTCGTCGATTTTTCCG-3'; ePTEN-R: 5'-GATCGCGGCCGC-CATTTCCCGAACGTCGATCAAAAC-3'. The PCR product was digested with Not1, ligated into pBluescript (Stratagene) and the plasmid DNA was bulk prepared (without cloning) after transformation into E. Coli. Germline transformation was performed as previously described [S2]. For rescue experiments, the pBluescript-PTEN/daf-18 vector was co-injected into daf-2(e1368) daf-18(e1375) double mutant animals at a concentration of 20 µg/ml with the dominant roller marker pRF4 (80 $\mu g/ml$). Transgenic roller animals were picked, and the phenotype was scored (roller adult versus roller dauer) after shifting the temperature from 15°C to 25.5°C.

References

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